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Kinetics of chlorobenzene biodegradation under reduced oxygen levels

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Abstract Focussing on the role of chlorocatechol 1,2-dioxygenase (CC12O), an oxygen-dependent key enzyme in the aerobic catabolism of chlorobenzene (CB), *Pseudomonas veronii* strain UFZ B549, *Acidovorax facilis* strain UFZ B530, and a community of indigenous groundwater bacteria were amended with CB degradation under either oxic or hypoxic conditions. All cultures readily degraded CB at high oxygen availability, but had differing abilities to completely degrade CB when exposed to oxygen limitation. For the three cultures very distinct oxygen half-saturation constants (0.3–11.7 μM) for the respective CC12Os were obtained and protein analysis showed that high affinity-type *A. facilis* and low affinity-type *P. veronii* express CC12Os, which belong to different structural clusters. From this a functional relation between CC12O type and the ability to cope with efficient ring fission under oxygen limitation is anticipated. Extremely high oxygen affinities for CC12Os support the assumption that truly oxic environments are not an essential requirement to degrade chloro(aromatic) compounds. Tiny quantities of oxygen permanently resupplied will sufficiently maintain the growth of microaerophilic specialists with the ability to transform chloro(aromatics) via catechol intermediates.

Keywords Hypoxic · Kinetics · Chlorobenzene · Optode · Chlorocatechol 1,2-dioxygenase · Oxygen affinity

Abbreviations

CB	Chlorobenzene
3CC	3-Chlorocatechol
CC12O	Chlorocatechol 1,2-dioxygenase

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Introduction

The presence of dissolved oxygen is a key factor for rapid turnover of many hydrocarbons. Yet, due to a number of chemical and biological oxygen demanding processes combined with limited oxygen re-supply oxic conditions (e.g., >60 μM dissolved oxygen, DO) are rarely met in sub-surface environments (Chapelle et al. 1996; Balcke et al. 2007). Since inter-phase mass transfer of atmospheric oxygen may lack behind the microbial oxygen consumption, dissolved oxygen concentrations actually achieved in solution can be significantly decreased, although oxygen is provided. Therefore, microorganisms developed strategies to cope with oxygen limitations. Typically, heterotrophic microorganisms compensate for hypoxic environments (≪60 μM DO) by (i) the expression of oxygen-requiring enzymes adapted to function at low oxygen availabilities as found for terminal oxidases (Alexeeva et al. 2003; Mendz et al. 2000) or for oxygenases (Krooneman et al. 1998; Kukor and Olsen 1996), by (ii) elevating the synthesis of such enzymes in response to oxygen limitation (Dikshit et al. 1990), or by (iii) synthrophic interactions in order to overcome enzymatic bottlenecks associated with the oxygen limitation (Kiesel et al. 2007).

During the aerobic degradation of chlorobenzene (CB), besides its function as terminal electron acceptor, oxygen serves as co-substrate for hydrocarbon activation and ring fission (Reineke 2001). The most abundant degradation pathway is initiated by CB dioxygenase, which consumes 1 mol of oxygen per mole of CB to produce CB-*cis*-1,2-dihydrodiol. Subsequently, by activity of CB-*cis*-1,2-dihydrodiol dehydrogenase, 3-chlorocatechol (3CC) is formed. Another mole of oxygen per mole of 3CC is required for the subsequent *ortho*-cleavage of 3CC formed to give *cis*,*cis*-2-chloromuconate, followed by chloride elimination and further breakdown reactions (Reineke and Knackmuss 1984; Schlömann, 1994).

The enzyme catalyzing the cleavage of 3CC is catechol 1,2-dioxygenase (CC12O; EC 1.13.11.1). CC12Os can be classified in three sub-families on the basis of sequence identities and substrate specificities: (i) enzymes with high affinities for catechol only or catechol and 4-methylcatechol, (ii) enzymes with relatively high affinities for 3- and 4-methylcatechols, and (iii) enzymes with high affinities for chlorocatechols (Murakami et al. 1997). The latter enzymes are active in degradation pathways of chlorinated aromatic compounds.

Although they are the key to rapid degradation, affinities of these dioxygenases towards oxygen and expression under hypoxic conditions have been sparsely described so far. The lowest reported oxygen half-saturation constants of catechol or chlorocatechol dioxygenases are clearly higher than those of terminal oxidases with values between 0.7–271 μM (Ferreira Jorge and Livingston 1999; Kukor and Olsen 1996; Nakai et al. 1990; Nakajima et al. 2002; Viliesid and Lilly 1992) and 0.007–0.35 μM, respectively (D'Mello et al. 1996; Preisig et al. 1996; Rice and Hempfling 1978), inferring a generally lower oxygen affinity of ring cleaving enzymes versus respiratory oxidases. Corroborated by the often observed (chloro)catechol accumulation at oxygen depletion, just the inadequate activity of the ring cleaving (chloro)catechol 1,2-dioxygenase or 2,3-dioxygenase enzymes is anticipated to limit further biodegradation of (chloro)aromatic compounds. Yet, an efficient catechol turnover is crucial to the fate of aromatic pollutants because accumulation of catechols is also toxic to bacteria (Schweigert et al. 2001).

Moreover, although in various bacteria several catechol or chlorocatechol dioxygenase iso-enzymes are encoded on the genome or on plasmids (Moiseeva et al. 2002; Nakai et al. 1990; Thiel et al. 2005), it has not been demonstrated yet that selective expression of alternative catechol dioxygenases is regulated by the oxygen concentration as it is known for terminal oxidases (Otten et al. 2001; Rice and Hempfling 1978; Tseng et al. 1996).

In this study, we address the degradation of chlorobenzene in view of different oxygen availability, comparing a bacterial groundwater community and two key organisms isolated earlier from sediment material of the CB-polluted groundwater from Bitterfeld, Germany, during a hydrogen peroxide treatment (Alfreider et al. 2002, 2003; Vogt et al. 2004a). The strains, A*cidovorax facilis* UFZ B530 and *Pseudomonas veronii* UFZ B549, use the modified *ortho*-pathway for degradation of CB as indicated by positive enzyme assays for chlorocatechol 1,2-dioxygenase, and negative enzyme assays for chlorocatechol 2,3-dioxygenase using CB-grown cells (Vogt et al. 2004b and unpublished results). The

rationale here was to prove whether mineralization of CB can be still achieved under severe oxygen limitation and to delineate the key role of CC12Os involved. We hypothesized that microorganisms that can compensate best for hypoxic conditions by CC12Os, which favorably function under oxygen-limited conditions, will display the highest mineralization rates. We tested whether, characteristic for a strain and according to the oxygen availability, different CC12O iso-enzymes would be employed.

Methods

Experimental setup

Duran glass bottles of 585 ml total volume filled with 325 ml chloride-poor mineral salt medium were used as described previously (Balcke et al. 2004). The bottles were equipped with a teflon-coated magnetic stir bar and sealed by fresh Mininert lids with extra 2 mm thick butyl septa (Supelco, Fig. 1a). During the experiment, the bottles were stored in a plastic bag continuously flushed with argon (5.0). Thus, undesired oxygen diffusion through the lid was proven to be negligible. Above and below the water table, oxygen sensitive optode spots (POF-PtSt3 and TOS7, Presens, Regensburg, Germany) were glued to the inside of each glass bottle (Fig. 1a). In an optode an embedded fluorophor can be excited from the exterior side of the glass wall by an external source of light. With oxygen present (detectable in gas and water) the fluorescence intensity is quenched, which is used to determine the interior oxygen concentration in a non-invasive manner (Klimant et al. 1997; Tolosa et al. 2002). Each spot was calibrated separately prior to sterilization of the glassware using air-saturated mineral medium and sodium dithionite. Evaluating all associated headspace and dissolved oxygen data pairs, and applying the ideal gas law, as well as a Henry constant of 0.00158 mol kg^{-1} bar^{-1} (15°C) (Lide and Friderikse 1995), the absolute oxygen contents of microcosms were derived for each time step. The cumulative oxygen demand was calculated from differences in absolute oxygen contents of consecutive oxygen concentration data pairs and was corrected for losses in total oxygen due to the withdrawal of samples for chemical analyses.

Oxygen-fed batch cultures

All experiments were conducted at 15 ± 2°C and in darkness. The stirring speed was fixed to 450 rpm.

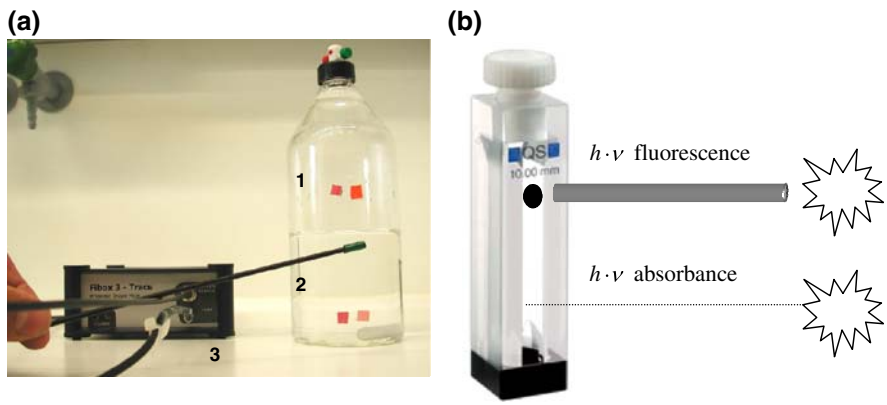

Fig. 1 Experimental design for oxygen measurement in fed batches (**a**) and enzyme assays (**b**). (**a**) A nearly constant oxygen partial pressure was maintained by discontinuous oxygen gas spikes. Oxygen sensitive optode spots (1) attached above and below the water table allowed for rapid analysis of oxygen in the gas and the water phase. A non-invasive ultra-sensitive oxygen measurement was achieved by optic detection of fluorescence light through the glass wall via light transducers (2) and a fiber-optic device (FIBOX 3 trace, Presens) (3). Tailored optode materials of different sensitivity allowed for obtaining signals specific to particular oxygen ranges (e.g., ultra trace range 60 nM–35 μM). Throughout this study, headspace oxygen partial pressures were converted into equilibrium dissolved oxygen saturations according to the Henry Law. For the sake of clarity, dissolved and headspace oxygen concentrations are both expressed in micromole per liter aqueous solution. (**b**) A half-micro quartz cuvette equipped with an optode spot and a mini magnetic stir bar was used to determine oxygen-dependent kinetic parameters of cell extracts in low volumes

Prior to the inoculation each bottle was thoroughly flushed with a mixture of sterile N_2/CO_2 that balanced a pH of 6.7, spiked either with 600 μM succinate or with neat CB to 600 μM (assuming all CB to be dissolved in the water phase), and equilibrated for 5 h to dissolve the CB. Sterile oxygen (5.0 grade, Linde AG, Germany) gas was spiked into the microcosms using a gastight syringe with cock (Hamilton, USA), 0.2 μm cellulose acetate filters (Millipore), and flame sterilized needles whenever the O_2 headspace concentration fell 1.75 μM (expressed as water saturation) below the desired level, thus maintaining oxygen concentrations of either about 11 μM (0.75% O_2, hypoxic) or 215 μM (14.4% O_2, oxic) in the headspace. Liquid samples of 500 μl were withdrawn using disposable syringes with a hot, flame-sterilized needle. The concentrations of CB, oxygen, metabolites, chloride, and protein were sampled periodically throughout the experiment. The experiment was carried out with one replicate for each bacterial culture and oxygen level, respectively. The endpoint of each batch was set when the oxygen concentrations in headspace and medium converged (no apparent oxygen demand).

Chemical analyses

Protein was measured according to a Bradford assay (Roti-Nanoquant, Roth, Germany) (Zor and Seliger 1996). Before measurements cell clusters were disrupted by intensive mixing for 1 min and treatment in an ultrasonic bath (HF 35 kHz) for 1 min. Metabolite analysis was carried out on a Dionex Summit HPLC which was equipped with diode array detector PDA100 and an Ultrasep ES Phenol 7MY column (Sepserv, Berlin, Germany). Eluents were (A) 1% acetic acid, 10% acetonitrile, and (B) 1% acetic acid, 99% acetonitrile. A gradient up to 90% solvent B was driven. 3CC and *cis*,*cis*-2-chloromuconic acid were available as authentic standards (Kaschabek and Reineke 1994). Identification was achieved by retention time and diode array spectrum. CB was determined by triplicate GC-FID analyses of 50 μl samples taken with a flamed syringe needle from the batch headspace (see Balcke et al. 2004 for other details). Chloride was measured by a Dionex ICS2000 system, column AS11HC.

Preparation of the inocula

An indigenous bacterial community from a CB polluted, virtually oxygen-free site at Bitterfeld, Germany was collected as described previously (Balcke et al. 2004). About 5 ml of the re-suspended filter pellet (in 100 ml medium, about 5 × cells ml^{-1}) were used as inoculum. The two pure strains, *A. facilis* and *P. veronii*, were precultured on CB (1 mM) in modified Brunner Medium (Vogt et al. 2004a), and cultivated either under fully oxic conditions (when subjected to oxic main experiments) or under conditions ≤10 μM oxygen saturation (for hypoxic main experiments) until the early stationary growth phase was reached. Cells were subsequently washed twice in substrate/chloride-free modified Brunner medium, concentrated afterwards to approximately 5×10^9 cells l^{-1}, and used as inoculum (2 ml) for batch cultures within 20 h.

Oxygen demand kinetics

Intact cells: Immediately at the end of batch cultivations the oxygen demand was re-induced by spiking CB to about 220 μM. An aliquot of the cell suspension was filled brimful into a half-micro quartz cuvette, avoiding the existence of any headspace by pressing a glass stopper into the overflowing suspension (Fig. 1b). During the transfer some oxygen (up to 75 μM) dissolved in the cell suspensions. The time-dependent oxygen decline was measured down to the detection limit of the oxygen meter (sensor TOS7, 60 nM). Cell extracts were produced by triplicate freeze–thawing (using liquid nitrogen and a water bath) and separation from oxidases, localized in the cell walls, was achieved by centrifugation at 12,000*g* for 8 min at 4°C. To minimize potential enzyme inactivation, cell concentrates (up to a hundredfold), obtained by centrifugation, were used. CC12O activities were determined according to Hegeman (1966) with 1 mM 3CC as excess substrate in 40 mM Tris–HCl, pH 8, and 1.3 mM EDTA whereas the oxygen consumption was determined by fluorescence detection (Fig. 1). Kinetic parameters for the oxygen depletion were obtained by numerical differentiation of the oxygen depletion curves, data arrangement to $d[O_2]/dt$ versus $[O_2]$ and non-linear regression using the Michaelis–Menten equation with

oxygen as substrate (Origin 7.5, OriginLab Corporation, Northhampton, MA, USA). Levenberg-Marquardt iteration was repeated until maximum convergence was achieved. Only fits with regression coefficients >0.92 were accepted to derive kinetic parameters.

Protein separation and identification

Cell extracts of *A. facilis* and *P. veronii* were prepared as described previously (Benndorf et al. 2004). About 50 μg of acetone precipitated protein were separated by SDS-PAGE (Laemmli 1970). Gels were stained with colloidal coomassie-brilliant blue and dried in a stream of unheated air. Protein bands of interest were excised and digested overnight with trypsin (Santos et al. 2004). The extracted peptides were separated by reversed-phase nano-LC (LC1100 series, Agilent Technologies, Palo Alto, California; column: Zorbax 300SB-C18, 3.5 μm, 150 × 0.075 mm; eluent: 0.1% formic acid, 0–60% acetonitrile) and analyzed by tandem mass spectrometry (LC/MSD TRAP XCT mass spectrometer, Agilent Technologies, Palo Alto, California). Database searches were carried out with MS/ MS ion search (MASCOT, http://www.matrixscience.com) against NCBInr.

Results and discussion

Interplay between oxygen phase transfer and biodegradation of CB

Although the headspace O_2 concentration was kept to equilibrium levels of approximately 11 μM (0.75% O_2 in the headspace, Fig. 2) and 210 μM (14.4% O_2, supplemental information), respectively, and a high stirring speed was applied, dissolved oxygen concentrations much lower than the equilibrium level were detected in the media. These dissolved oxygen levels are being in continuous steady state between oxygen re-supply and consumption, which is determined by the overall activity of all oxygen requiring enzymes in the medium. Complete aeration would have been possible within 110 min (supplementals). Thus, microbial oxygen consumption rates clearly exceeded the rate of the O_2 phase transfer, as even for cultures grown under oxic conditions dissolved oxygen concentrations fell below equilibrium levels. Distinct steady state dissolved oxygen concentrations reflected a different ability of individual cultures to cope with oxygen limitation (Fig. 2). Differences between replicates were observed only as temporal delay of the CB catabolism (maximum 2 days), whereas the DO and metabolite levels were comparable throughout. Therefore, only representative examples are discussed in the following. *Acidovorax facilis* B530. Nearly constant dissolved oxygen concentrations of about 2–3 μM prevailed during the first 5 days (Fig. 2a) although the protein concentration in the media increased progressively by 2 μg ml^{-1} (data not shown). Within these 5 days CB was nearly completely converted but with concomitant accumulation of 3CC and large quantities of the ring fission product *cis*,*cis*-2-chloromuconate. After 5 days, when CB was less than 20 μM, the concentration of 2-chloromuconate also decreased, accompanied by concomitant chloride formation and a distinct decline of dissolved oxygen from 2–3 μM to a lower level near 1 μM. Ongoing oxygen demand during this phase accompanied the transformation of intermediates even after CB had been depleted virtually completely. The oxygen consumption terminated when *cis*,*cis*-2-chloromuconate was entirely transformed. Within 10 days the chloride release reached high values close to 100% of the theoretical value.

Although under oxygen limitation *Pseudomonas veronii* B549 was also able to transform CB, less dehalogenation and observation of brown polycondensates of 3CC (Farrell and Quilty 1999) suggest a slow conversion of 3CC (Fig. 2b). As an indication of a toxification occurring, decreasing oxygen demand caused increasing oxygen concentrations as observed from the beginning to day 4. Yet, the time-course of the cumulative oxygen demand reflects that the oxygen consumption was not fully suppressed. Consequently, steady state dissolved oxygen concentrations in the media equilibrated to higher levels (8 μM, days 4–7) in comparison with *A. facilis*. During this phase only little chloride was released although the CB transformation continued. Concomitant with slow CB depletion, the dissolved oxygen level decreased only slowly accompanied by moderate chloride release (Fig. 2b, days 9–14). In contrast to *A. facilis*, hypoxic batches with *P. veronii* turned

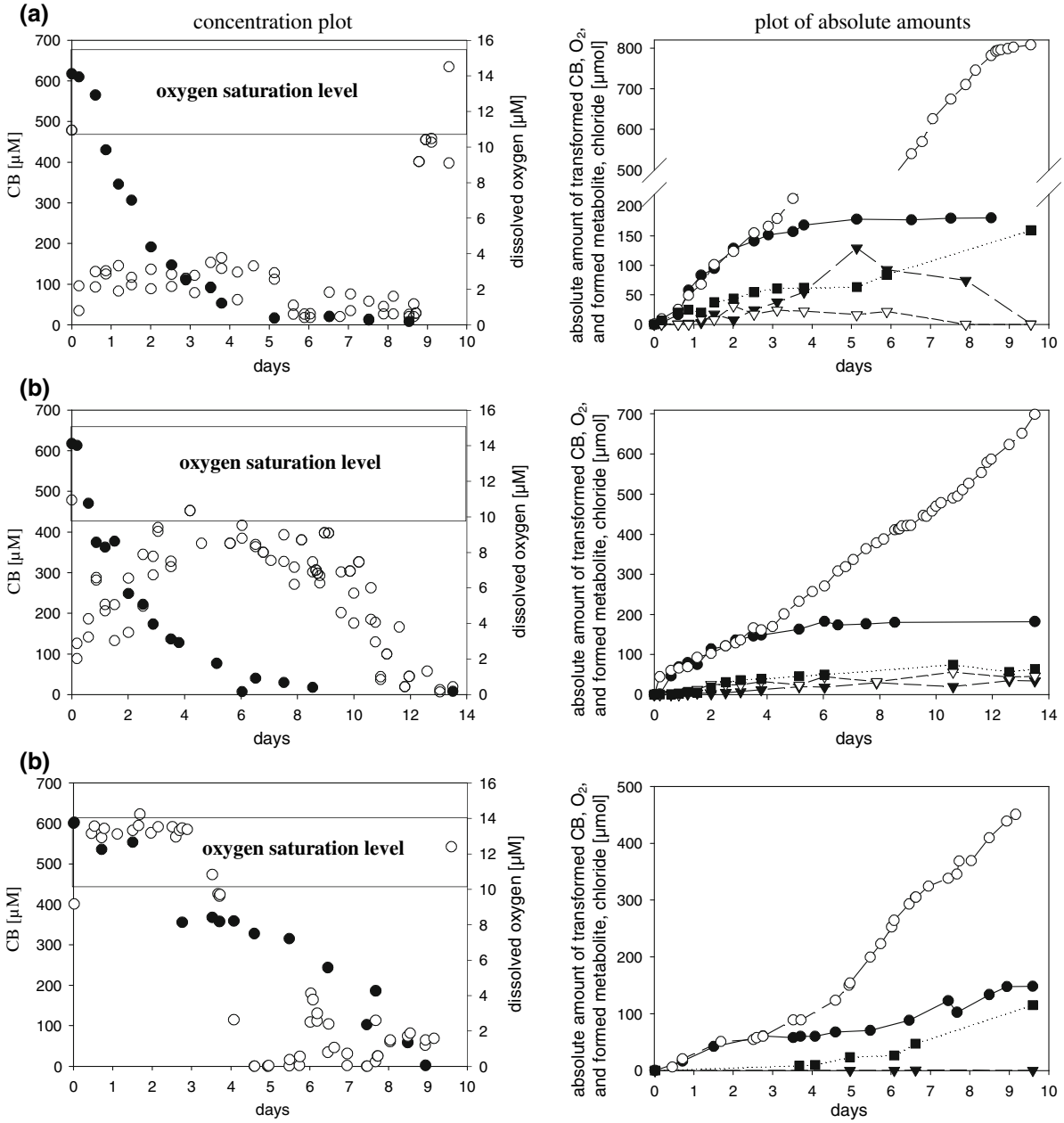

Fig. 2 Chlorobenzene (CB) biodegradation under hypoxic conditions in representative replicates of (**a**) *A. facilis* B530, (**b**) *P. veronii* B549, and (**c**) a Bitterfeld groundwater consortium. Constantly low oxygen concentrations in the headspace of the microcosms were maintained by numerous oxygen gas re-spikes. For clarity, all measured headspace oxygen concentrations are given as a range (oxygen saturation level). (**a**) and (**b**) were adapted to hypoxic conditions prior to the inoculation. Left: CB, dissolved oxygen concentration versus incubation time, and oxygen saturation level (frame). Right: Total amounts of CB transformed, cumulative amounts of oxygen consumed, *cis*,*cis*-2-chloromuconate and 3-chlorocatechol formed, and chloride released. ● CB, ○ O_2, ■ chloride, ▼ *cis*,*cis*-2-chloromuconate (CM), ▽ 3-chlorocatechol

brown, displayed low protein formation, and only 40% of the theoretical dehalogenation after a 2-week incubation (Table 1).

The turnover of CB by *a fresh groundwater consortium* was characterized by a slow initial transformation (lag phase). Again, first 1 mol oxygen

Table 1 Degradation parameters obtained at the end of individual hypoxic and oxic fed-batch experiments

Culture	Hypoxic					Oxic				
	Incubation days	CB transformed (%)	Chloride released (%)	Final ratio (O_2/CB)	Protein formed (mg l^{-1})	Incubation (days)	CB transformed (%)	Chloride released (%)	Final ratio (O_2/CB)	Protein formed (mg l^{-1})
Groundwater consortium	11.6	100*	77.7 (80.4)	2.6 (3.4)	1.2 (1.4)	4.9	100	87.7 (89.2)	11.2 (12.6)	9.0 (9.3)
Acidovorax facilis B530	9.6	100*	84.3 (80.3)	4.4 (3.7)	5.3 (5.6)	1.6	100	84.3 (79.0)	6.0 (6.2)	6.4 (6.5)
Pseudomonas veronii B549	13.5	100*	39.7 (37.0)	3.8 (3.4)	0.2 (0.2)	1.5	100	92.0 (83.3)	4.4 (5.4)	4.1 (7.2)

Values in parentheses give the results of a replicate experiment

*Initial concentration 620 μM CB

was consumed per mole CB transformed as would have been expected for chlorocatechol formation (Fig. 2c), which, however, was not observed in the medium. Steady state dissolved oxygen concentrations did not decrease immediately to low values. But when the oxygen demand per mole CB converted exceeded an equimolar ratio (day 3), the dissolved oxygen concentrations declined steeply to $\ll$1 μM (Fig. 2c). This effect was associated with an interim halt in further CB transformation and a sub-stoichiometric chloride release (days 4–6). Similar oxygen levels were observed earlier during oxygen-limited growth of several cultures on succinate degradable without need of dioxygenase reactions (Nestler et al. 2007). Therefore, it is presumed that dissolved oxygen levels $\ll$1 μM were associated with the activity of terminal oxidases, whereas sufficient CC12O activity could not be maintained at this level. Subsequently, accompanied by an intermittent increase of the dissolved oxygen concentration (days 6–7) the CB conversion recovered whereupon the dissolved oxygen adjusted to a level near 2 μM as observed for *A. facilis*. Monitoring changes in the composition of the groundwater bacterial community in response to oxygen limitation was beyond the scope of this study, but is addressed in detail by Kiesel et al. (2007).

Distinct steady state dissolved oxygen concentration levels as emerging during wide ranges of hypoxic cultivations are a measure for the overall activity of all oxygen consuming enzymes. Comparable cell densities and headspace oxygen partial pressures given, lower steady state dissolved oxygen concentrations reflect higher enzyme activities. But in turn, lower steady state dissolved oxygen levels also induce higher oxygen re-supply (Fig. 2). Hence, by expression of high affinity-type CC12Os microaerophilic specialists can set steady state oxygen levels to low oxygen concentrations, which are not suitable for bacteria with low affinity for oxygen to maintain similar enzyme activities. In conclusion, when characteristic steady state oxygen levels can be assigned to the activity of CC12Os specific to a particular culture, potential changes in the community composition can be explained in terms of the ability of specialists to better transform toxic chlorocatechols under conditions where oxygen is limiting. This hypothesis was tested for consortia by Kiesel et al. (2007) and Nestler et al. (2007).

Oxygen demand kinetics

The cleavage of 3CC in all cultures investigated was catalyzed by chlorocatechol 1,2-dioxygenase, as proved by enzyme tests of cell-free extracts (Vogt et al. 2004b) and protein expression analysis (see below).

For individual cultures nearly identical half-saturation constants were obtained irrespective if the CC12O activity was determined directly on cell extracts and 3CC as substrate, or if intact cells were exposed to CB in short term. However, when kinetic parameters of different cultures are compared, considerable differences between the cultures become evident and apparent oxygen half-saturation constants differed by a factor of 39 (Table 2). Three representative examples for non-linear regression of experimental data to the Michaelis–Menten equation are illustrated in Fig. 3.

A*cidovorax facilis* displayed similarly low oxygen half-saturation constants of 2.5–2.8 μM independently of the oxygen availability during the previous cultivation (i.e., oxic or hypoxic) and comparably low maximum specific oxygen uptake rates were measured (Table 2). In comparison, for *P. veronii* much higher apparent oxygen half-saturation constants of about 11 μM were derived. Yet, maximum specific oxygen uptake rates were more than five times those of *A. facilis* (Table 2). An oxygen half saturation constant of 11.6 μM in the course of chlorobenzene degradation during continuous cultivation was also reported for *Pseudomonas* sp. JS150 (Ferreira Jorge and Livingston 1999), indicating that the organism uses a CC12O with similar properties as *P. veronii*.

In contrast to both pure cultures, very different kinetic parameters for oxic and respective hypoxic cultivations of the *groundwater consortium* point at community shifts as a result of an adaptation to the oxygen availability. While extremely low apparent oxygen half-saturation constants (0.3 μM) characterized the oxygen demand kinetics of hypoxic cultures, low oxygen affinities similar to those of *P. veronii* were obtained from oxic cultures (Table 2). Nonetheless, intact cell kinetics and enzyme tests on CC12O using crude cell extracts gave again nearly equal oxygen half-saturation constants.

Tests on whole cells (where simultaneously all oxygen consuming enzymes might be activated) and on crude extracts (where only membrane-bound oxidases are removed) do not allow for direct comparison of specific CC12O activities. However, the close match of oxygen half-saturation constants determined with intact cells on CB and cell-free extracts on 3CC (Table 2), the rapid initial CB oxidation to 3CC within a wide range of oxygen tensions (Fig. 2 and supplementals), and the equimolar initial ratio of consumed oxygen and CB (Fig. 2) clearly indicate that in particular the activity of CC12O limits the oxygen demand during oxygen-limited CB conversion (Fig. 2, Tables 1 and 2). Moreover, different steady state dissolved oxygen levels observed for three different cultures (*A. facilis* 2.4 μM in average between 0 and 5 days, *P. veronii*: 6.9 μM 0–9 days, groundwater culture: 1.1 μM 4–9 days) correspond to the respective oxygen

Table 2 Kinetic parameters for catechol 1,2-dioxygenase from intact cells and crude cell extracts of representative hypoxic and oxic cultures

Culture	Hypoxic		Oxic			
	Intact cells (CB as substrate)		Intact cells (CB as substrate)		Cell extracts (3-chlorocatechol as substrate)	
	V_{max} (μM O_2 min^{-1} mg $protein^{-1}$)	K_m (O_2) (μM)	V_{max} (μM O_2 min^{-1} mg $protein^{-1}$)	K_m (O_2) (μM)	V_{max} (nM O_2 min^{-1} mg $protein^{-1}$)	K_m (O_2) (μM)
Groundwater consortium	2.4 (2.6)	0.3 (0.3)	0.5 (0.4)	7.7 (11.1)	2.7 (3.1)	11.4 (11.4)
Acidovorax facilis B530	0.2 (0.3)	2.8 (2.7)	0.3 (0.3)	2.5 (2.7)	1.1 (1.1)	2.6 (2.6)
Pseudomonas veronii B549	n.d.*	n.d.*	1.6 (1.6)	11.7 (11.6)	11.5 (11.5)	10.0 (10.1)

Values in parentheses give the results of a replicate experiment

*Kinetic determinations for hypoxic batches of this strain were omitted because these cultures exhibited inhibition kinetics due to accumulation of toxic 3-chlorocatechol

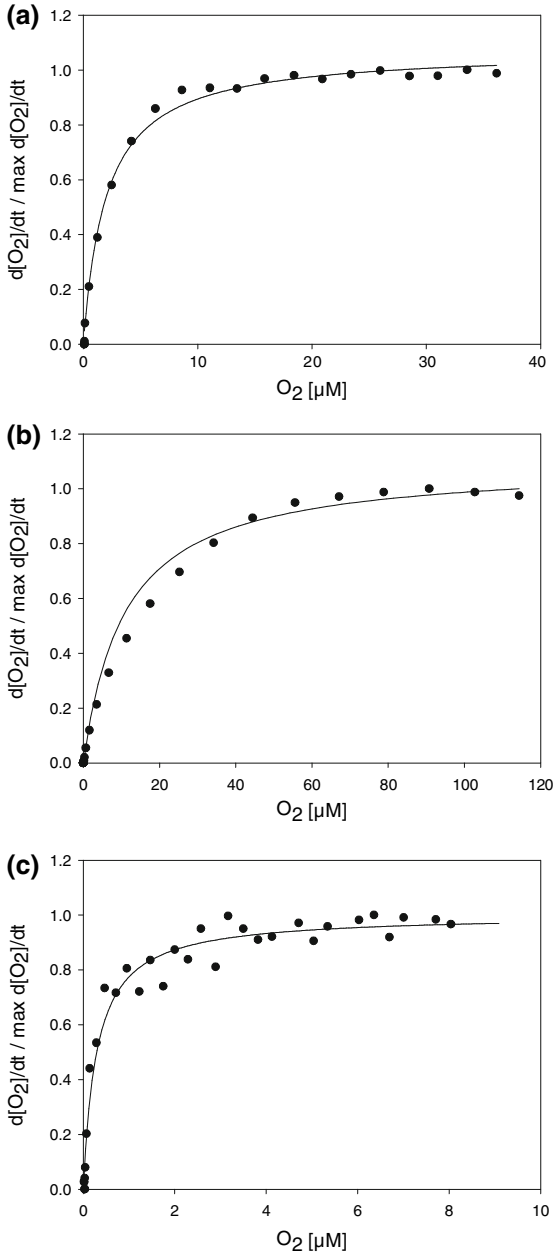

Fig. 3 Normalized oxygen transformation rates versus dissolved oxygen for intact cells of (**a**) *A. facilis* B530 (oxic cultivation), (**b**) *P. veronii* B549 (oxic cultivation), and (**c**) a hypoxic Bitterfeld groundwater consortium exposed to excess CB concentrations. Dotted: measured data; line: non-linear regression using the Michaelis–Menten equation

half-saturation, which corroborates the assumption that primarily the activity of CC12O determines steady state oxygen levels over a wide time-course.

Protein expression analysis

Compared to growth on succinate (non-induced), growth of *A. facilis* and *P. veronii* on CB under oxic and hypoxic conditions caused the induction of CC12Os (Fig. 4). Although there are some proteins differing between oxic and hypoxic growth of each individual strain, we identified several proteins involved in the catechol pathway induced by both oxic and hypoxic conditions (not shown). In order to locate the chlorocatechol 1,2-dioxygenases in the gel, we decided to cut bands in the range of 25–35 kDa (Fig. 4), since the calculated molecular weight of chlorocatechol 1,2-dioxygenases is about 30 kDa (http://www.expasy.org/). In both strains, only one single chlorocatechol 1,2-dioxygenase was identified by mass spectrometry of tryptic peptides. The molecular masses of chlorocatechol 1,2-dioxygenases in *A. facilis* and *P. veronii* are different from each other. The enzyme of *A. facilis* has a molecular mass of 30 kDa. Masses of the peptides (precursor ions) and of corresponding fragments matched best to chlorocatechol 1,2-dioxygenase of *Delftia acidovorans* (AAC35836.2) and *Achromobacter xylosoxidans* (CAD56206.2). Aligned peptide fragments covered 46% of the data base entries with 100% identity (Fig. 5). The chlorocatechol 1,2-dioxygenase of *P. veronii* is larger (35 kDa) and the data from mass spectrometry best matched to chlorocatechol 1,2-dioxygenases of *Ralstonia* sp. JS705 (CAO6968.1), *Pseudomonas aeruginosa* (AAF00195.1), and *Pseudomonas putida* (CAE92861.1), (37% sequence coverage with 100% identity, Fig. 5).

Although the technique used does not allow for quantitative determination of enzyme expressions, it could be demonstrated that *A. facilis* and *P. veronii* express individual CC12Os, which show homology to distinct sub-clusters of chlorocatechol 1,2-dioxygenases (Hoffmann et al. 2003). Both clusters contain mostly CC12Os from β-proteobacteria (*Burkholderiales*) or γ-proteobacteria (*Pseudomonadales*) that are encoded on the chromosome or on plasmids and were probably distributed by horizontal gene transfer. If both enzymes are assumed to be identical with the matched proteins from *Ralstonia* sp. JS705 and *Delftia acidovorans* P4a, respectively, the chlorocatechol 1,2-dioxygenases of *A. facilis* and *P. veronii* have only about 65% identical positions. Yet, equal apparent oxygen half-saturation constants for oxic

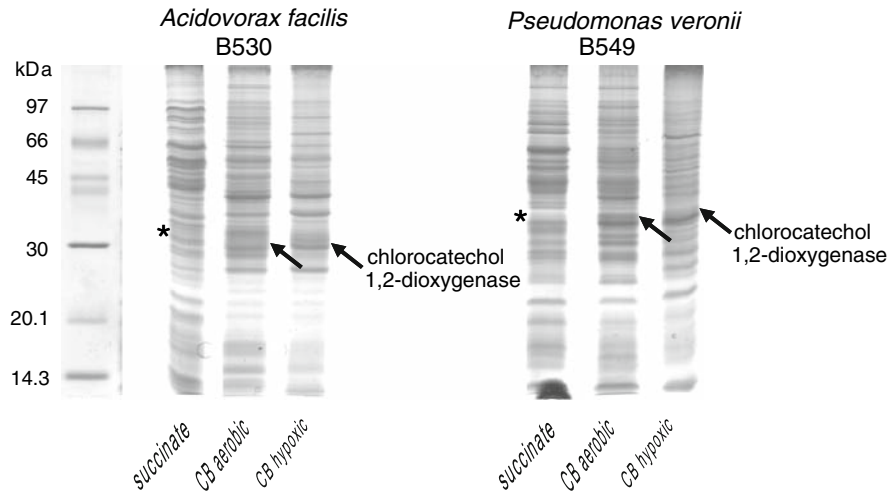

Fig. 4 Protein expression pattern of *A. facilis* B530 and *P. veronii* B549 after growth on succinate (non-induced) or after growth on CB (induced) under oxic and hypoxic conditions. CC12Os identified by mass spectrometry are marked with arrows. Asterisks: positions of gel slices cut from protein pattern of succinate grown cells as negative controls

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Ralstonia sp. JS705        MDKRVAEVAG AIVEAVRKIL LDKRVTEAEY RAGVDYLTEV AQTRETALLL
Delftia acidovorans P4a    MNERVKQVAS ALVDAIQKTL TEQRVTEEEW RAGVGYMMKL AEAKEMDLLL
Consensus sequence         M#.RV.#VA. A.V#A!.K.L .#.RVTE.E. RAGV.Y$... A#..E..LLL

                           51                                                100
Ralstonia sp. JS705        DVFLNSTIIE GKAWRSRTSA PAIQGPYFLE GAPVVEGVLK TYDTDDHKPL
Delftia acidovorans P4a    DVFFNHTIVD LKAQATRGST PAIQGPYFLE GAPVVAGALK TYEDDSHHPL
Consensus sequence         DVF.N.TI!# .KA...R.S. PAIQGPYFLE GAPVV.G.LK TY#.D.H.PL

                           101                                               150
Ralstonia sp. JS705        IIRGTVRSDT GELLAGAVID VWHSTPDGLY SGIHDNIPVD YYRGKLVTDS
Delftia acidovorans P4a    VIRGAVRTDD GAPAAGAVID VWHSTPDGKY SGFHDQIPTD LYRGKVVADA
Consensus sequence         !IRG.VR.D. G...AGAVID VWHSTPDG.Y SG.HD#IP.D .YRGK.V.D.

                           151                                               200
Ralstonia sp. JS705        QGNFRVRTTM PVPYQIPYEG PTGRLLGHLG SHTWRPAHVH FKVRKDGFEP
Delftia acidovorans P4a    QGKYAVRTTM PAPYQIPNKG PTGVLLEMMG SHTWRPAHVH FKVRKDGFVP
Consensus sequence         QG.%.VRTTM P.PYQIP..G PTG.LL..$G SHTWRPAHVH FKVRKDGF.P

                           201                                               250
Ralstonia sp. JS705        LTTQYYFEGG KWVDDDCCHG VTPDLITPET IEDGVRVMTL DFVIEREQAE
Delftia acidovorans P4a    LTTQYYFEGG DWVDSDCCKG VAPDLVMPAK TEGGAQVMAI DFVIERPREH
Consensus sequence         LTTQYYFEGG .WVD.DCC.G V.PDL!.P.. .E.G..VM.. DFVIER....

                           251    260
Ralstonia sp. JS705        QRKSATETVA
Delftia acidovorans P4a    A
Consensus sequence         ..........
```

Fig. 5 Alignment of catechol 1,2-dioxygenases from *Ralstonia* sp. JS705 and *Delftia acidovorans* P4a and the respective consensus level. Bold letters indicate peptides covering the sequences of *P. veronii* B549 (not underlined) to *Ralstonia* sp. JS705 and *A. facilis* B530 (underlined) to *Delftia acidovorans* P4a as detected by tandem-mass spectrometry. Multalin version 5.4.1 (Multiple sequence alignment with hierarchical clustering (Corpet, 1988); Symbol comparison table: blosum62, Gap weight: 12, Gap length weight: 2, Consensus levels: high = 90%; Consensus symbols: ! is anyone of IV, $ is anyone of LM, % is anyone of FY, # is anyone of NDQEBZ)

and hypoxic cultivations of both pure strains (Table 2), protein expression patterns, and sequence identifications suggest that the investigated strains of *A. facilis* and *P. veronii* did not express different chlorocatechol 1,2-dioxygenases due to different oxygen availability. Thus, unlike different terminal oxidases, which can be selectively expressed according to the oxygen availability, expression of isoenzymes according to the oxygen availability was not observed.

On the basis of kinetic and sequence data presented, fundamental enzymological differences

of CC12Os from high-affinity type (*A. facilis*) and from low-affinity type (*P. veronii*) strains are anticipated. The facts that the CC12Os of *A. facilis* and *P. veronii* differ in oxygen affinity and show homology to distinct clusters as observed previously in cDNA amplificates of key organisms in the Bitterfeld groundwater (Alfreider et al. 2003) raises the question whether the oxygen affinity of CC12Os is a property, which is common to each individual cluster. Further elucidation of structure–function relations of (chloro)catechol dioxygenases is indicated, since the catechol cleavage of aromatic structures is decisive for the degradability of many xenobiotics.

The apparent oxygen half-saturation constant of 0.3 μM reflects to our knowledge the highest oxygen affinity of a catechol or intradiol dioxygenase ever published. Moreover, the value of 0.3 μM DO deduced in this study is low enough to match with the lowest reported oxygen affinities of terminal oxidases (0.35 μM, 0.2 μM Rice and Hempfling 1978). Hence, extremely low apparent oxygen half-saturation constants for key enzymes involved in the degradation of CB support the assumption that truly oxic environments are not an essential requirement to degrade chloro(aromatic) compounds. A low quantity of oxygen permanently re-supplied will sufficiently maintain the growth of microaerophilic specialists with the ability to express high levels of CC12Os at low oxygen availability.

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References

Alexeeva S, Hellingwerf KJ, de Mattos MJT (2003) Requirement of ArcA for redox regulation in *Escherichia coli* under microaerobic but not anaerobic or aerobic conditions. J Bacteriol 185:204–209

Alfreider A, Vogt C, Babel W (2002) Microbial diversity in an *in situ* reactor system treating monochlorobenzene contaminated groundwater as revealed by 16S ribosomal DNA analysis. Syst Appl Microbiol 25:232–240

Alfreider A, Vogt C, Babel W (2003) Expression of chlorocatechol 1,2-dioxygenase and chlorocatechol 2,3-dioxygenase genes in chlorobenzene-contaminated subsurface samples. Appl Environ Microbiol 69:1372–1376

Balcke GU, Turunen LP, Geyer R, Wenderoth DF, Schlosser D (2004) Chlorobenzene biodegradation under consecutive aerobic–anaerobic conditions. FEMS Microbiol Ecol 49:1–12

Balcke GU, Meenken S, Hoefer C, Oswald SE (2007) Kinetic gas-water transfer and gas accumulation in porous media during pulsed oxygen sparging. Environ Sci Technol 41(12):4428–4434

Benndorf D, Davidson I, Babel W (2004) Regulation of catabolic enzymes during long-term exposure of *Delftia acidovorans* MC1 to chlorophenoxy herbicides. Microbiology 150:1005–1014

Chapelle FH, Haack SK, Adriaens P, Henry MA, Bradley PM (1996) Comparison of E(h) and H-2 measurements for delineating redox processes in a contaminated aquifer. Environ Sci Technol 30:3565–3569

Corpet F (1988) Multiple sequence alignment with hierarchical clustering. Nucleic Acid Res 16(22):10881–10890

Dikshit KL, Dikshit RP, Webster DA (1990) Study of *Vitreoscilla* globin (vgb) gene-expression and promoter activity in *E. coli* through transcriptional fusion. Nucleic Acids Res 18:4149–4155

D'Mello R, Hill S, Poole RK (1996) The cytochrome bd quinol oxidase in *Escherichia coli* has an extremely high oxygen affinity and two oxygen-binding haems: implications for regulation of activity in vivo by oxygen inhibition. Microbiology 142:755–763

Farrell A, Quilty B (1999) Degradation of mono-chlorophenols by a mixed microbial community via a meta-cleavage pathway. Biodegradation 10:353–362

Ferreira Jorge RM, Livingston AG 1999. A novel method for characterisation of microbial growth kinetics on volatile organic compounds. Appl Microbiol Biotechnol 52: 174–178

Hegeman GD (1966) Synthesis of the enzymes of the mandelate pathway by *Pseudomonas putida*. I. Synthesis of enzymes by the wild type. J Bacteriol 91:1140–1154

Hoffmann D, Kleinsteuber S, Müller RH, Babel W (2003) A transposon encoding the complete 2,4-dichlorophenoxyacetic acid degradation pathway in the alkalitolerant strain *Delftia acidovorans* P4a. Microbiology 149:2545–2556

Kaschabek SR, Reineke W (1994) Synthesis of bacterial metabolites from haloaromatic degradation. 1. Fe(III)-catalyzed peracetic-acid oxidation of halocatechols, a facile entry to *cis*,*cis*-2-halo-2,4-hexadienedioic acids and 3-halo-5-oxo-2(5H)-furanylideneacetic acids. J Org Chem 59:4001–4003

Kiesel B, Balcke GU, Dietrich J, Vogt C, Geyer R (2007) Microbial community shifts as a response to efficient degradation of chlorobenzene under hypoxic conditions. Biodegradation, doi: 10.1007/s10532-007-9149-z

Klimant I, Kuhl M, Glud RN, Holst G (1997) Optical measurement of oxygen and temperature in microscale: strategies and biological applications. Sens Actuat B Chem 38:29–37

Krooneman J, Moore ERB, van Velzen JCL, Prins RA, Forney LJ, Gottschal JC (1998) Competition for oxygen and 3-chlorobenzoate between two aerobic bacteria using different degradation pathways. FEMS Microbiol Ecol 26:171–179

Kukor JJ, Olsen RH (1996) Catechol 2,3-dioxygenases functional in oxygen-limited (hypoxic) environments. Appl Environ Microbiol 62:1728–1740

Laemmli UK (1970) Cleavage of structural proteins during assembly of head of Bacteriophage-T4. Nature 227: 680–685

Lide DR, Frederikse HPR (eds) (1995) CRC handbook of chemistry and physics, 76th edn. CRC Press, Inc., Boca Raton, FL

Mendz GL, Smith MA, Finel M, Korolik V (2000) Characteristics of the aerobic respiratory chains of the microaerophiles *Campylobacter jejuni* and *Helicobacter pylori*. Arch Microbiol 174:1–10

Moiseeva O, Solyanikova IP, Kaschabek SR, Groning J, Thiel M, Golovleva LA, Schlömann M (2002) A new modified ortho cleavage pathway of 3-chlorocatechol degradation by *Rhodococcus opacus* 1CP: genetic and biochemical evidence. J Bacteriol 184:5282–5292

Murakami S, Kodama N, Shinke R, Aoki K (1997) Classification of catechol 1,2-dioxygenase family: sequence analysis of a gene for the catechol 1,2-dioxygenase showing high specificity for methylcatechols from Gram+ aniline-assimilating *Rhodococcus erythropolis* AN-13. Gene 185:49–54

Nakai C, Horiike K, Kuramitsu S, Kagamiyama H, Nozaki M (1990) Three isoenzymes of catechol 1,2-dioxygenase (pyrocatechase), $\alpha\alpha$, $\alpha\beta$, and $\beta\beta$, from *Pseudomonas arvilla*. J Biol Chem 265:660–665

Nakajima H, Ishida T, Tanaka H, Horiike K (2002) Accurate measurement of near-micromolar oxygen concentrations in aqueous solutions based on enzymatic extradiol cleavage of 4-chlorocatechol: applications to improved low-oxygen experimental systems and quantitative assessment of back diffusion of oxygen from the atmosphere. J Biochem 131:523–531

Otten MF, Stork DR, Reijnders WNM, Westerhoff HV, Van Spanning RJM (2001) Regulation of expression of terminal oxidases in *Paracoccus denitrificans*. Eur J Biochem 268:2486–2497

Nestler H, Kiesel B, Kaschabek SR, Mau M, Schlömann M, Balcke GU (2007) Biodegradation of chlorobenzene under hypoxic and mixed hypoxic-denitrifying conditions. Biodegradation doi: 10.1007/s10532-007-9104-z

Preisig O, Zufferey R, ThonyMeyer L, Appleby CA, Hennecke H (1996) A high-affinity cbb(3)-type cytochrome oxidase terminates the symbiosis-specific respiratory chain of *Bradyrhizobium japonicum*. J Bacteriol 178:1532–1538

Reineke W (2001) Biodegradation and persistence. In: The handbook of environmental chemistry, vol 2, Part K. Springer, Heidelberg, Berlin

Reineke W, Knackmuss HJ (1984) Microbial metabolism of haloaromatics: isolation and properties of a chlorobenzene-degrading bacterium. Appl Environ Microbiol 47:395–402

Rice CW, Hempfling WP (1978) Oxygen-limited continuous culture and respiratory energy-conservation in *Escherichia coli*. J Bacteriol 134:115–124

Santos PM, Benndorf D, Sa-Correia I (2004) Insights into *Pseudomonas putida* KT2440 response to phenol-induced stress by quantitative proteomics. Proteomics 4: 2640–2652

Schlömann M (1994) Evolution of chlorocatechol catabolic pathways. Biodegradation 5:301–321

Schweigert N, Zehnder AJB, Eggen RIL (2001) Chemical properties of catechols and their molecular modes of toxic action in cells, from microorganisms to mammals. Environ Microbiol 3:81–91

Thiel M, Kaschabek S, Groning J, Mau M, Schlömann M (2005) Two unusual chlorocatechol catabolic gene clusters in *Sphingomonas* sp. TFD44. Arch Microbiol 183: 80–94

Tolosa L, Kostov Y, Harms P, Rao G (2002) Noninvasive measurement of dissolved oxygen in shake flasks. Biotechnol Bioeng 80:594–597

Tseng CP, Albrecht J, Gunsalus RP (1996) Effect of microaerophilic cell growth conditions on expression of the aerobic (cyoABCDE and cydAB) and anaerobic (narGHJI, frdABCD, and dmsABC) respiratory pathway genes in *Escherichia coli*. J Bacteriol 178:1094–1098

Viliesid F, Lilly MD (1992) Influence of dissolved oxygen tension on the synthesis of catechol 1,2 diooxygenase by *Pseudomonas putida*. Enzyme Microb Technol 14: 561–565

Vogt C, Alfreider A, Lorbeer H, Hoffmann D, Wünsche L, Babel W (2004a) Bioremediation of chlorobenzene-contaminated groundwater in an in situ reactor mediated by hydrogen peroxide. J Cont Hydrol 68:121–141

Vogt C, Simon D, Alfreider A, Babel W (2004b) Microbial degradation of chlorobenzene under oxygen-limited conditions leads to accumulation of 3-chlorocatechol. Environ Toxicol Chem 23:265–270

Zor T, Selinger Z (1996) Linearization of the Bradford protein assay increases its sensitivity: theoretical and experimental studies. Anal Biochem 236:302–308